PATENT
Docket No.: **19603/2986 (CRF D-1940B)**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Qiu et al.	Examiner: Anne R. Kubelik
Serial No.	:	09/766,348	
Cnfrm. No.	:	7683	Art Unit: 1638
Filed	:	January 19, 2001	
For	:	HYPERSENSITIVE RESPONSE INDUCED RESISTANCE IN PLANTS BY SEED TREATMENT	

APPEAL BRIEF

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P.O. Box 1450
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Dear Sir:

Pursuant to 37 CFR § 41.37, appellants hereby file their appeal brief. Enclosed is the filing fee of $500.00 required by 37 CFR § 41.20(b)(2). You are hereby authorized to charge/credit Deposit Account No. 14-1138 for any deficiency/overage.

I. REAL PARTY IN INTEREST

Cornell Research Foundation, Inc., as assignee of U.S. Patent Application Serial No. 09/766,348 (referred to herein as "the '348 Application"), is the real party in interest.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences pertaining to the above-identified application.

III. STATUS OF CLAIMS

A. Claims 41, 49-51, 53, 58-61, 69-71, 73, 75-77, 80, 82, and 84 Are Finally Rejected

Claims 41, 49-51, 53, 58-61, 69-71, 73, 75-77, 80, 82, and 84 have been rejected under 35 U.S.C. § 112 (1st para.) for failure to comply with the written description requirement.

B. Claims 1-40, 42-48, 52, 54-57, 62-68, 72, 74, 78, 79, 81, 83, and 85 Have Been Canceled

Claims 1-40, 42-48, 52, 54-57, 62-68, 72, 74, 78, 79, 81, 83, and 85 have been canceled.

C. No Claims Stand Allowed

No claims stand allowed.

D. Claims 41, 49-51, 53, 58-61, 69-71, 73, 75-77, 80, 82, and 84 Are On Appeal

The decision of the Examiner finally rejecting claims 41, 49-51, 53, 58-61, 69-71, 73, 75-77, 80, 82, and 84 is appealed. These claims, in their currently pending form, are set forth in the attached **Claims Appendix**.

IV. STATUS OF AMENDMENTS

Appellants filed an Amendment Under 37 C.F.R. § 1.116 on March 29, 2005. As reflected in the April 20, 2005, Advisory Action, this Amendment was to be entered for purposes of appeal. Thus, there are no amendments pending.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The present invention is directed to a method of imparting pathogen resistance to plants by providing a transgenic plant seed transformed with a transgene containing a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein (page 11, line 31 to page 12, line 5, and page 13, lines 1-4 of the '348 Application). The method also involves planting the transgenic plant seed in soil (page 12, lines 2-3, and page 13, lines 4-5 of the '348 Application) and propagating a plant from the planted seed (page 12, lines 3-5, and page 13, lines 5-7 of the '348 Application). In accordance with this method, expression of the

hypersensitive response elicitor polypeptide or protein by the plant imparts systemic pathogen resistance to the plant (page 12, lines 3-5 and 12 of the '348 Application). The encoded hypersensitive response elicitor polypeptide or protein has an amino acid sequence of SEQ ID NO:1 (page 16, line 9 to page 17, line 25 of the '348 Application), SEQ ID NO:3 (page 18, line 51 to page 20, line 10 of the '348 Application), SEQ ID NO:5 (page 21, line 24 to page 22, line 35 of the '348 Application), or SEQ ID NO:7 (page 23, line 38 to page 24, line 46 of the '348 Application). The transgene also contains a promoter that is not pathogen-inducible (page 36, lines 17-21 of the '348 Application). The promoter is operatively coupled to the DNA molecule encoding the hypersensitive response elicitor polypeptide or protein (page 36, lines 14-21 of the '348 Application).

The present invention is also directed to a method of imparting pathogen resistance to plants by transforming a plant with a transgene containing a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein (page 11, line 31 to page 12, line 5, page 13, lines 1-4, page 33, line 31, and page 36, lines 9-30 of the '348 Application). The transforming of the plant provides for expression of the hypersensitive response elicitor polypeptide or protein that imparts systemic pathogen resistance to the plant (page 12, lines 3-5 and 12, page 33, line 31, and page 36, lines 9-30 of the '348 Application). The encoded hypersensitive response elicitor polypeptide or protein has an amino acid sequence of SEQ ID NO:1 (page 16, line 9 to page 17, line 25 of the '348 Application), SEQ ID NO:3 (page 18, line 51 to page 20, line 10 of the '348 Application), SEQ ID NO:5 (page 21, line 24 to page 22, line 35 of the '348 Application), or SEQ ID NO:7 (page 23, line 38 to page 24, line 46 of the '348 Application). The transgene also contains a promoter that is not pathogen-inducible (page 36, lines 17-21 of the '348 Application). The promoter is operatively coupled to the DNA molecule encoding the hypersensitive response elicitor polypeptide or protein (page 36, lines 14-21 of the '348 Application).

The present invention is further directed to a transgenic plant produced by a process which involves transforming a plant with a transgene containing a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein (page 11, line 31 to page 12, line 5, page 13, lines 1-4, page 33, line 31, and page 36, lines 9-30 of the '348 Application). The transforming of the plant provides for expression of the hypersensitive response elicitor polypeptide or protein that imparts systemic pathogen resistance to the plant (page 12, lines 3-5 and 12, page 33, line 31, and page 36, lines 9-30 of the '348 Application). The encoded hypersensitive response elicitor polypeptide or protein has an amino acid sequence of SEQ ID NO:1 (page 16, line 9 to page 17, line 25 of the '348 Application), SEQ

ID NO:3 (page 18, line 51 to page 20, line 10 of the '348 Application), SEQ ID NO:5 (page 21, line 24 to page 22, line 35 of the '348 Application), or SEQ ID NO:7 (page 23, line 38 to page 24, line 46 of the '348 Application). The transgene also contains a promoter that is not pathogen-inducible (page 36, lines 17-21 of the '348 Application). The promoter is operatively coupled to the DNA molecule encoding the hypersensitive response elicitor polypeptide or protein (page 36, lines 14-21 of the '348 Application).

VI. GROUNDS OF REJECTION TO BE REVIEWED

Whether claims 41, 49-51, 53, 58-61, 69-71, 73, 75-77, 80, 82, and 84 are properly rejected under 35 U.S.C. § 112 (1st para.) for failure to satisfy the written description requirement, where the present application clearly teaches the use of non-inducible promoters (including constitutive promoters).

VII. ARGUMENT

A. <u>Applicable Law—35 U.S.C. § 112 (1st paragraph)</u>

The "written description" requirement under 35 U.S.C. § 112 (1st para.) has been held to be distinct from the "enablement" requirement of this same section. *See Vas-Cath v. Mahurkar*, 935 F.2d 1555, 1563, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991). The purpose of the "written description" requirement is to ensure that the inventor had possession of the invention claimed at the time the application was filed. *Id.* To achieve this, the application must in some manner describe the invention with all its claimed limitations. *See Lockwood v. American Airlines, Inc.*, 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997); *In re Wertheim*, 541 F.2d 257, 262, 191 USPQ 90, 96 (CCPA 1979). If new matter is added to the claims, the claims may be subject to rejection under the written description requirement of 35 U.S.C. § 112 (1st para.). *In re Rasmussen*, 650 F.2d 1212, 211 USPQ 323 (CCPA 1981).

Determining whether the description requirement is met must be done on a case-by-case basis and is a question of fact. *In re Wertheim*, 541 F.2d at 262, 191 USPQ at 96 (CCPA 1976). The description, as filed, is presumed to be adequate, unless or until sufficient evidence or reasoning to the contrary has been presented by the examiner to rebut the presumption. *In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971). In instances in which an explicit limitation in a claim "is not present in the written description whose benefit is sought[,] it must be shown that a person of ordinary skill would have

understood, at the time the patent application was filed, that the description requires that limitation." *Hyatt v. Boone*, 146 F.3d 1348, 1353, 47 USPQ2d 1128, 1131 (Fed. Cir. 1998)).

B. The Rejection of Claims 41, 49-51, 53, 58-61, 69-71, 73, 75-77, 80, 82, and 84 Under 35 U.S.C. § 112 (1st para.) for Failure to Satisfy the Written Description Requirement Is Improper

The Examiner has taken the position that neither the specification nor the originally filed claims provide support for the phrase "promoter that is not pathogen-inducible" (as recited in pending claims 41, 61, and 75). For the reasons set forth below, this rejection is improper. More than sufficient written descriptive support exists in the present application for the recited claim language at issue.

The following passage appears in the present application at page 36, lines 17-21:

> ***As is conventional in the art***, such transgenic plants would contain suitable vectors with ***various promoters*** including pathogen-induced promoters, and other components needed for transformation, transcription, and, possibly, translation.

(emphasis added). The clear meaning of this language is that "various promoters" can be used to make the claimed transgenic plants. One type of promoter that falls within the class of "various promoters" is said to be "pathogen-induced promoters." However, the above passage clearly does not limit the "various promoters" to such "pathogen-induced promoters"; pathogen-induced promoters are one example of suitable promoters. In the universe of "various promoters" where "pathogen-induced promoters" are an example, the rest of that universe of "various promoters" must, as a simple matter of logic, be the claimed non-pathogen-inducible promoters. This is entirely consistent with the knowledge that those skilled in the art of transgenic plants would have possessed at the time the present invention was made.

At the time the present invention was made, one of ordinary skill in the art was well aware of the use of constitutive and other non-inducible promoters for transforming plants. *See* Koncz et al., "The Opine Synthase Genes Carried by Ti Plasmids Contain All Signals Necessary for Expression in Plants," *EMBO J.* 2(9):1597-1603 (1983) (referred to herein as "Koncz") (attached hereto as **Exhibit 1**); U.S. Patent No. 5,034,322 to Rogers et al. (referred to herein as "Rogers '322") (attached hereto as **Exhibit 2**); and U.S. Patent No. 5,352,605 to Fraley et al. (referred to herein as "Fraley '605") (attached hereto as **Exhibit 3**). Thus, the phrase "various promoters" in the specification would have been understood by

those skilled in the art to encompass, besides pathogen-induced promoters, promoters that are *not* pathogen-inducible (e.g., constitutive promoters).

Koncz was published over 17 years before the filing of the present application, and identifies the nopaline synthase ("NOS") promoter from *Agrobacterium tumefaciens*. As described in more detail below, at the time the present invention was made, it was well known that the NOS promoter had been used to successfully transform plant cells with chimeric genes. It is well known in the art that the NOS promoter is *not* a pathogen-induced promoter, but rather is a constitutive promoter. Thus, the NOS promoter qualifies as a promoter that is *not* pathogen-inducible.

Rogers '322 issued as a U.S. patent on July 23, 1991, nearly 10 years before the filing of the present application. Rogers '322 describes chimeric genes that are capable of being expressed in plant cells (col. 7, lines 18-20). These chimeric genes are said to have been used to create antibiotic-resistant plant cells and as being useful for creating herbicide-resistant plants and plants that contain mammalian polypeptides (Abstract; col. 7, lines 59-64; col. 9, lines 22-25). In a preferred embodiment, the chimeric genes are described as including the NOS promoter from *Agrobacterium tumefaciens* (col. 7, lines 21-29; col. 9, lines 16-17). Rogers '322 also states that "[o]ther suitable promoter regions may be derived from genes which exist naturally in plant cells" (col. 7, lines 29-31). For example, in other preferred embodiments, Rogers '322 teaches making chimeric genes using a promoter region taken from a gene which naturally exists in soybean (i.e., the gene in soybean that codes for the small subunit of ribulose-1,5-bis-phosphate carboxylase) (col. 16, line 48 to col. 18, line 43).

Fraley '605 issued as a U.S. patent on October 4, 1994, over six years before the filing of the present application. Fraley '605 describes chimeric genes for transforming plant cells using viral promoters (col. 3, lines 21-23). In a particular embodiment, Fraley '605 describes using the 35S promoter or the 19S promoter from cauliflower mosaic virus ("CaMV") to make chimeric genes that have been proven to be expressed in plant cells (col. 3, lines 26-37; col 4, line 1 to col. 8, line 62). It is well known in the art that the 35S and 19S promoters are *not* pathogen-induced promoter, but rather are constitutive promoters. Thus, the 35S and 19S promoters qualify as promoters that are *not* pathogen-inducible. Fraley '605 also described using the NOS promoter for constructing chimeric genes for transforming plants (col. 8, line 66 to col. 13, line 51).

Thus, Koncz, Rogers '322, and Fraley '605 constitute strong evidence that the present application intended to cover the use of pathogen-inducible and non-pathogen-inducible promoters in transgenic plants.

In making the final rejection, the Examiner states that above-quoted page 36, lines 17-21 of the present application shows that "at the time of filing, the only promoters contemplated were pathogen-induced promoters or promoters in general, which included pathogen-induced ones" (page 3 of the Final Office Action, mailed October 29, 2004). Appellants submit that this does not comport with what one of ordinary skill in the art would understand from reading the specification and is an unduly narrow view of the above-quoted passage. As noted above, the specification teaches making transgenic plants from a genus of "various promoters," with one example being pathogen-induced promoters. Given the knowledge in the art that non-pathogen-inducible promoters are useful in transgenic plants, one of ordinary skill in the art would not simply construe the specification as only teaching the use of pathogen-inducible promoters or promoters generally. Having taught that pathogen-induced promoters are just an example of suitable "various promoters," the other promoters which would constitute suitable various promoters would have to be the well known non-pathogen-inducible promoters.

The Examiner's position in the Advisory Action that the specification's recitation of "various promoters including pathogen-induced promoters" does not provide support for any specific type of promoter *other than* pathogen-inducible promoters (page 2 of the Advisory Action, mailed the April 20, 2005) is even further off-target. Apparently, in the Examiner's view, the specification teaches *only* using pathogen-inducible promoters to transform plants with the hypersensitive response elicitors of the present application (*Id.*). In particular, the Examiner asserts that, "[a]t the time of filing, the only promoters contemplated were pathogen-induced promoters or promoters in general, which included pathogen-induced ones" (*Id.*). Appellants completely disagree. Nowhere does the specification limit the claimed promoter to ***only*** a pathogen-inducible promoter.

For the foregoing reasons, it is submitted that appellants were in possession of the claimed invention at the time they filed the present application. Therefore, the rejection of claims 41, 49-51, 53, 58-61, 69-71, 73, 75-77, 80, 82, and 84 under 35 U.S.C. § 112 (1st para.) for failure to satisfy the written description requirement is improper and should be withdrawn.

VIII. CONCLUSION

In view of the foregoing, it is clear that the rejection of the claims under 35 U.S.C. § 112 (1st para.) cannot be sustained. Accordingly, the rejection should be reversed.

Respectfully submitted,

Dated: December 2, 2005

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December 2, 2005
Date

Jo Ann Whalen

IX. CLAIMS APPENDIX

41. A method of imparting pathogen resistance to plants, the method comprising:

providing a transgenic plant seed transformed with a transgene comprising a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein comprising an amino acid sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:7 and a promoter that is not pathogen-inducible, the promoter being operatively coupled to the DNA molecule encoding the hypersensitive response elicitor polypeptide or protein;

planting the transgenic plant seed in soil; and

propagating a plant from the planted seed, whereby expression of the hypersensitive response elicitor polypeptide or protein by the plant imparts systemic pathogen resistance to the plant.

49. The method according to claim 41, wherein the plant is selected from the group consisting of dicots and monocots.

50. The method according to claim 49, wherein the plant is selected from the group consisting of rice, wheat, barley, rye, oats, cotton, sunflower, canola, peanut, corn, potato, sweet potato, bean, pea, chicory, lettuce, endive, cabbage, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane.

51. The method according to claim 49, wherein the plant is selected from the group consisting of rose, *Saintpaulia,* petunia, *Pelargonium*, poinsettia, chrysanthemum, carnation, and zinnia.

53. The method according to claim 41 further comprising:

applying the hypersensitive response elicitor polypeptide or protein to the plant to enhance the plant's pathogen resistance.

58. A plant produced by the method of claim 41.

59. A transgenic plant seed from the plant produced by the method of claim 41, wherein the transgenic plant seed comprises the transgene.

60. A plant propagule from the plant produced by the method of claim 41.

61. A method of imparting pathogen resistance to plants, the method comprising:

transforming a plant with a transgene comprising a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein comprising an amino acid sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:7 and a promoter that is not pathogen-inducible, the promoter being operatively coupled to the DNA molecule encoding the hypersensitive response elicitor polypeptide or protein, whereby said transforming provides for expression of the hypersensitive response elicitor polypeptide or protein that imparts systemic pathogen resistance to the plant.

69. The method according to claim 61, wherein the transgenic plant is selected from the group consisting of dicots and monocots.

70. The method according to claim 69, wherein the plant is selected from the group consisting of rice, wheat, barley, rye, oats, cotton, sunflower, canola, peanut, corn, potato, sweet potato, bean, pea, chicory, lettuce, endive, cabbage, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane.

71. The method according to claim 69, wherein the plant is selected from the group consisting of rose, Saintpaulia, petunia, Pelargonium, poinsettia, chrysanthemum, carnation, and zinnia.

73. The method according to claim 61, further comprising:

applying the hypersensitive response elicitor polypeptide or protein to the transgenic plant to enhance the plant's pathogen resistance.

75. A transgenic plant produced by a process comprising:

transforming a plant with a transgene comprising a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein comprising an amino acid sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:7 and a promoter that is not pathogen-inducible, the promoter being operatively coupled to the DNA molecule encoding

the hypersensitive response elicitor polypeptide or protein, whereby said transforming provides for expression of the hypersensitive response elicitor polypeptide or protein to impart systemic pathogen resistance to the transgenic plant.

76. A transgenic plant seed obtained from the transgenic plant of claim 75, wherein the transgenic plant seed comprises the transgene.

77. A transgenic plant propagule obtained from the transgenic plant of claim 75.

80. The method according to claim 41, wherein the DNA molecule comprises a nucleotide sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, or SEQ ID NO:8.

82. The method according to claim 61, wherein the DNA molecule comprises a nucleotide sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, or SEQ ID NO:8.

84. The transgenic plant according to claim 75, wherein the DNA molecule comprises a nucleotide sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, or SEQ ID NO:8.

X. EVIDENCE APPENDIX

A. **EXHIBIT 1 - Koncz et al., "The Opine Synthase Genes Carried by Ti Plasmids Contain All Signals Necessary for Expression in Plants," *EMBO J.* 2(9):1597-1603 (1983)**

- Introduced into the record by appellants on August 13, 2004, and considered by the Examiner in the office action, dated October 29, 2004.

B. **EXHIBIT 2 - U.S. Patent No. 5,034,322 to Rogers et al.**

- Discussed in appellants' Amendment Under 37 CFR § 1.116, dated March 29, 2005, and considered by the Examiner in the Advisory Action, dated April 20, 2005.

C. **EXHIBIT 3 - U.S. Patent No. 5,352,605 to Fraley et al.**

- Discussed in appellants' Amendment Under 37 CFR § 1.116, dated March 29, 2005, and considered by the Examiner in the Advisory Action, dated April 20, 2005.

The EMBO Journal Vol.2 No.9 pp.1597–1603, 1983

The opine synthase genes carried by Ti plasmids contain all signals necessary for expression in plants

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Signals necessary for *in vivo* expression of Ti plasmid T-DNA-encoded octopine and nopaline synthase genes were studied in crown gall tumors by constructing mutated genes carrying various lengths of sequences upstream of the 5′ initiation site of their mRNAs. Deletions upstream of position −294 did not interfere with expression of the octopine synthase gene while those extending upstream of position −170 greatly reduced the gene expression. The estimated size of the octopine synthase promoter is therefore 295 bp. The maximal length of 5′ upstream sequences involved in the *in vivo* expression of the nopaline synthase gene is 261 bp. Our results also demonstrated that Ti plasmid-derived sequences contain all signals essential for expression of opine synthase genes in plants. Expression of these genes, therefore, is independent of the direct vicinity of the plant DNA sequences and is not activated by formation of plant DNA and T-DNA border junction.

Key words: Agrobacterium tumefaciens/Ti plasmids/opine synthase genes/promoter regions

Introduction

Crown gall, a neoplastic disease of dicotyledonous plants, develops after infection of wounded tissue with *Agrobacterium tumefaciens* strains carrying large tumor-inducing (Ti) plasmids (Zaenen *et al.*, 1974; Van Larebeke *et al.*, 1974; Watson *et al.*, 1975). A well-defined segment (T-region) of the Ti plasmid is transferred and covalently integrated, without rearrangements, in plant nuclear DNA (Chilton *et al.*, 1977, 1980; Schell *et al.*, 1979; Thomashow *et al.*, 1980; Lemmers *et al.*, 1980; Zambryski *et al.*, 1980; Yadav *et al.*, 1980; Willmitzer *et al.*, 1980). The transferred DNA (T-DNA) is transcribed (Drummond *et al.*, 1977; Willmitzer *et al.*, 1981a; Gelvin *et al.*, 1981) by the host RNA polymerase II (Willmitzer *et al.*, 1981b).

Transformed crown gall cells are capable of autonomous growth in the absence of exogenous phytohormones (Braun, 1956). Moreover, these plant tumors synthesize a variety of low mol. wt. metabolites (termed opines) which are characteristic for Ti plasmid-induced tumors (Bomhoff *et al.*, 1976), and can be specifically metabolized by agrobacteria growing on the incited tumors (Petit *et al.*, 1970; Petit and Tempé, 1978; Schell *et al.*, 1979; Tempé *et al.*, 1980). The Ti plasmids are currently classified into three groups according to the type of opine they induce in the incited tumors as octopine, nopaline or agropine Ti plasmids (Guyon *et al.*, 1980).

The T-DNA in octopine tumors consists of two distinguishable segments: TL-DNA and TR-DNA (Thomashow *et al.*, 1980; De Beuckeleer *et al.*, 1981). TL-DNA, which is essential and sufficient for octopine crown gall formation, codes for eight polyadenylated transcripts, each expressed from an individual promoter (Gelvin *et al.*, 1982; Willmitzer *et al.*, 1982). One of these transcripts (transcript 3) was shown to code directly for the enzyme octopine synthase (Schröder *et al.*, 1981). The nucleotide sequence of this gene was elucidated and both the 5′ and the 3′ ends of the transcript were precisely identified by S1 nuclease mapping (De Greve *et al.*, 1982). The 5′ end of the transcript coding for octopine synthase is located close to the right border of TL-DNA at a distance of 350–400 bp. This gene is transcribed from right to left (Willmitzer *et al.*, 1982).

The T-DNA of nopaline Ti plasmids codes for up to 13 polyadenylated transcripts (Bevan and Chilton, 1982; Willmitzer *et al.*, 1983). The region responsible for tumor maintenance is highly homologous between octopine TL-DNA and nopaline T-DNA (Engler *et al.*, 1981). Transcripts and gene functions determined by this conserved 'core' region are common in octopine and nopaline tumors (Joos *et al.*, 1983; Willmitzer *et al.*, 1983). Two different opines were detected in nopaline tumors: agrocinopine (Ellis and Murphy, 1981) and nopaline (Petit *et al.*, 1970). The nopaline synthase gene has been localized by genetic and transcript mapping on *Hin*dIII fragment 23 of plasmids pTiC58 and pTiT37 (Holsters *et al.*, 1980; Hernalsteens *et al.*, 1980; Joos *et al.*, 1983; Willmitzer *et al.*, 1983). DNA sequencing of *Hin*dIII fragment 23 localized the nopaline synthase gene (Depicker *et al.*, 1982) and the precise position of the right T-DNA borders within *Hin*dIII fragment 23 (Zambryski *et al.*, 1982).

To determine whether all signals essential for the expression of the opine synthase genes *in vivo* are located between the 5′ initiation site of the opine genes and the junction site with plant DNA or whether expression of these genes is activated by plant DNA sequences, we constructed octopine and nopaline synthase genes with different lengths of sequences upstream of the 5′ initiation site and reinserted them in the T-DNA of the Ti plasmids. This approach allowed us to delimit which sequences are important for the *in vivo* expression of the octopine and nopaline synthase genes, and to demonstrate that the plasmid-derived sequences contain all signals necessary for expression in plants.

Results

Expression of the octopine synthase gene in nopaline tumors

Construction of intermediate vectors pGV761, pGV762 and pGV763. The precise number of base pairs in the DNA region between the 5′ initiation site of the octopine synthase transcript (De Greve *et al.*, 1982) and the right T-region border sequence (Holsters *et al.*, 1983) has been determined and was found to be 402 (Figure 1a). Therefore, sequences essential for the expression of octopine synthase must either be located in this sequence, or activation of the promoter occurs by junction of the 5′ end of the *ocs* gene with plant

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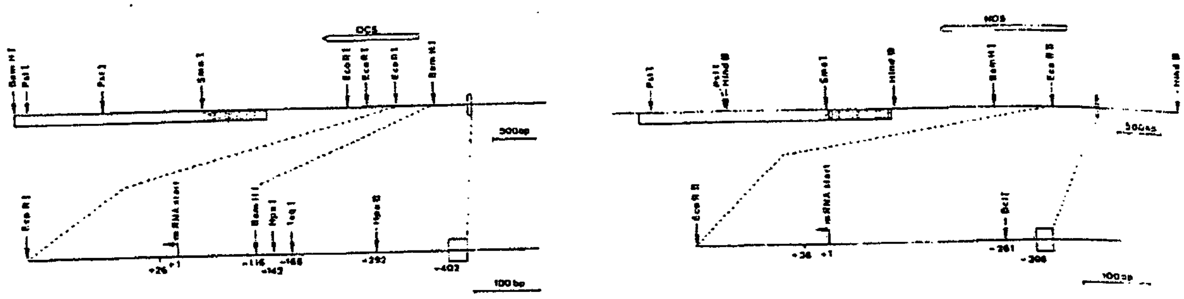


Fig. 1. (A) In the upper part the *Bam*HI fragment 17a and sequences up to the border (white box) are indicated, and the location and transcription polarity of the octopine synthase gene. The white bar shows the homology region between *Bam*HI fragment 17a and the nopaline T-DNA. The hatched portion of the white bar shows the homology region of 750 bp between plasmids pGV761, pGV762, pGV763 and the nopaline Ti plasmid. In the lower part the position of the restriction sites used in this study are indicated with regard to the transcription start of the octopine synthase gene. (B) In the upper part the *Hind*III fragments 23 and 31, and part of the *Hind*III fragment 22, are indicated (Depicker *et al.*, 1980). The position and transcription polarity of the nopaline synthase gene located in *Hind*III fragment 23 and the homology region with *Bam*HI fragment 17a of the octopine Ti plasmid pTiAch5 are shown. In the lower part the position of the *Bcl*I site is indicated with regard to the transcription start of the nopaline synthase gene.

DNA.

To test which of these possibilities is valid, intermediate vectors containing the octopine synthase gene and different lengths of 5′-flanking sequences (respectively −116 bp, −168 bp and −292 bp from the transcription start; Figure 1a) were constructed and introduced into the nopaline Ti plasmid C58. If the first possibility is correct, these constructions should allow us to delimit the sequences involved in the *in vivo* expression of the octopine synthase gene. The different steps in the construction of the intermediate vectors are outlined in Figure 2.

Isolation of co-integrated Ti plasmids. As the homology region between plasmids pGV761, pGV762 and pGV763 (Figure 1), and the nopaline Ti plasmid is only 750 bp, we envisaged, to avoid problems of recombination, using the homology of 1270 bp between the *amp* gene located on pBR322 and the transposon Tn*1*, inserted into the T-DNA of the nopaline Ti plasmid C58 (Joos *et al.*, 1983; Inzé *et al.*, in preparation).

For this purpose, we selected the plasmids pGV3300 and pGV3305. In pGV3300 a Tn*1* is inserted in *Hind*III fragment 23 just outside the nopaline synthase gene, while in pGV3305 the Tn*1* insertion is located in the nopaline synthase gene. The intermediate vectors pGV761, pGV762 and pGV763 were mobilized from *Escherichia coli* to *Agrobacterium* strains GV3101 (pGV3300) and GV3101 (pGV3305) with the helper plasmids R64*drd*11 and pGJ28 (Van Haute *et al.*, 1983). In all cases, Km^R transconjugants were isolated with a frequency of $10^{-6}-10^{-7}$. Several co-integrate plasmids resulting from a single cross-over were analyzed by DNA/DNA hybridization to confirm their physical structure (data not shown). Recombination always occurred within the homology region common to pBR322 and Tn*1*.

Properties of the co-integrated plasmids. Sunflower hypocotyls and tobacco W38 plants were inoculated with the *Agrobacterium* strains containing these different co-integrates. The different primary tumor tissues were subsequently analyzed for octopine synthase activity (Otten and Schilperoort, 1978). No octopine synthase activity was detected in sunflower and tobacco tumors induced by the *Agrobacterium* strains containing the co-integrated plasmids pGV2290 (pGV3300::pGV761) and pGV2291 (pGV3305::pGV761). Furthermore, in tumors induced by *Agrobacterium* strains containing the co-integrated plasmids pGV2292 (pGV3300::pGV762) and pGV2293 (pGV3305::pGV762), again no detectable octopine synthase activity could be detected. On the contrary, in sunflower and tobacco tumors induced with *Agrobacterium* strains containing the co-integrated plasmids pGV2294 (pGV3300::pGV763) and pGV2295 (pGV3305::pGV763), octopine synthase activity was detected (Figure 3). The level of activity in these tumors was equal to that found in tumors induced by the *Agrobacterium* strain C58C1 containing an octopine Ti plasmid (pTiB6S3Tra^C).

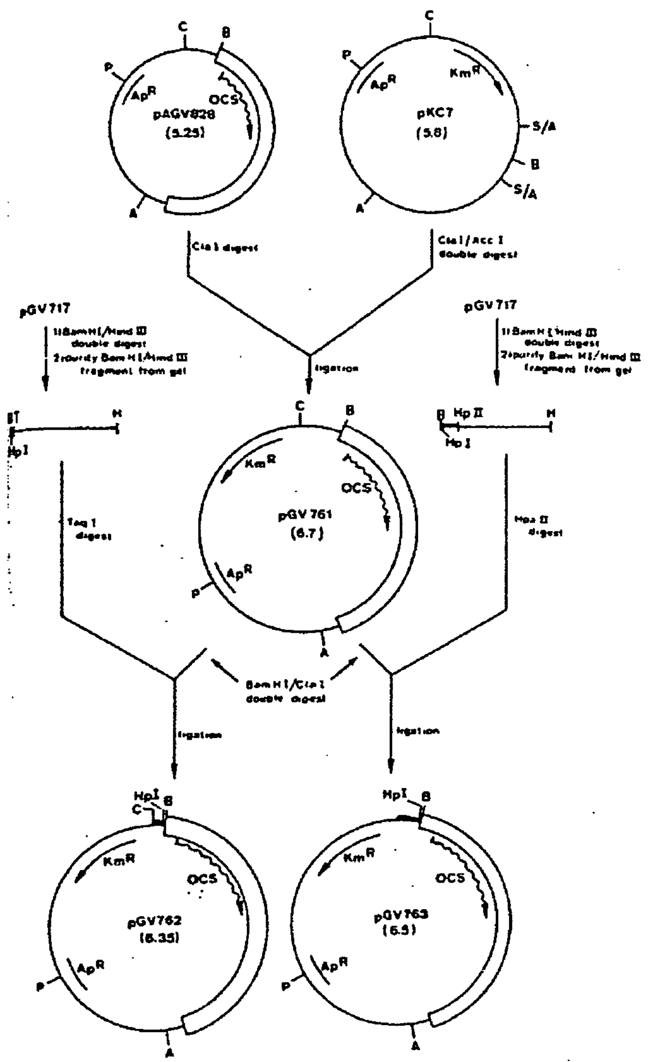
Expression of the nopaline synthase gene in octopine tumors

We have studied the expression of the nopaline synthase gene by a similar approach. DNA sequence analysis showed that the nopaline synthase gene is entirely encoded by the *Hind*III fragment 23 of pTiC58 (Depicker *et al.*, 1982). Furthermore, genomic blotting analysis of nopaline tumor tissues (Lemmers *et al.*, 1980) showed that this *Hind*III-23 fragment is a border fragment. Genomic clones isolated from different nopaline tumor tissues (Zambryski *et al.*, 1980, 1982; Holsters *et al.*, 1982) allowed us to determine the exact end point of the T-DNA in crown gall lines. The right T-DNA/plant DNA border is located only 305 bp (Figure 1b) from the start of the nopaline synthase transcript (Depicker *et al.*, 1982).

Construction and properties of pGV2253 and pGV2254

Construction of intermediate vectors pGV705 and pGV706. To demonstrate that the expression of the nopaline synthase gene is independent of the formation of a junction to plant DNA sequences, and that all sequences involved in the *in vivo* expression of the nopaline synthase gene are present between the start of the mRNA and the end of the T-DNA, we constructed an intermediate vector in which the sequences between the *Hind*III site and the *Bcl*I site (position −261; Figure 1b) of the *Hind*III fragment 23 have been deleted and replaced by the Sm^R gene of R702. This substitution deletes the 22-bp consensus sequence (position −30[illegible]; Figure 1b) which is found at the ends of nopaline and octopine T-regions, and which might play a key role in the integration of the T-region into the plant genome (Zambryski *et al.*, 1980, 1982; Simpson *et al.*, 1982; Yadav *et al.*, 19[illegible]; Holsters *et al.*, 1982, 1983). The construction of the intermediate vector pGV705 is shown in Figure 4.

pGV705 consists of *Eco*RI fragment 12 of pTiAch5 [illegible] which the internal *Hind*III-36a fragment has been substitu[illegible]



ig. 2. Construction of intermediate vectors pGV762 and pGV763. The cl-*Cla*I fragment of pKC7 containing the Km gene was ligated to *Cla*I-gested pAGV828. After ligation and selection on ApKm plates, recombinants were screened for the orientation of the Km-resistant fragment by uble digestion with *Cla*I and *Bam*HI. A recombinant plasmid pGV761 as digested with *Bam*HI and *Cla*I, and ligated to the purified *Hind*III-mHI fragment of pGV717, which contains sequences 5′ upstream of the mHI site at −116 in the promoter region of the octopine synthase gene igure 1; Holsters *et al.*, 1983), digested with either *Taq*I or *Hpa*II. By reening recombinant plasmids for the presence of a *Hpa*I site (Figure 1), V762 and pGV763 were obtained. Abbreviations: A, *Acc*I; B, *Bam*HI; *Cla*I; H, *Hind*III; HpI, *Hpa*I; HpII, *Hpa*II; P, *Pst*I; S, *Sal*I; T, *Taq*I.

the *Hind*III-*Bcl*I fragment of the nopaline *Hind*III fragent 23 joined to the *Bam*HI-*Hind*III fragment of plasmid 702 containing the Sm^R gene. This *Hind*III fragment inted in the other orientation in the *Eco*RI fragment 12, is lled pGV706.

Isolation of pGV2253 and pGV2254. The intermediate vecs pGV705 and pGV706 were mobilized from *E. coli* to robacterium strain GV3000 carrying a transfer-constitutive B6S3 plasmid with the help of the plasmids R64*drd*11 and J28 (Van Haute *et al.*, 1983). Streptomycin-resistant *Agroterium* strains were obtained in both cases with a joint nsfer and recombination frequency of 10^{-6}. The Smistant transconjugants were tested directly for Km sensitivity. Three percent of the Sm^R transconjugants were Km-sensitive and were double recombinants. The structure of two plasmids pGV2253 and pGV2254 was confirmed by DNA-DNA hybridization (data not shown).

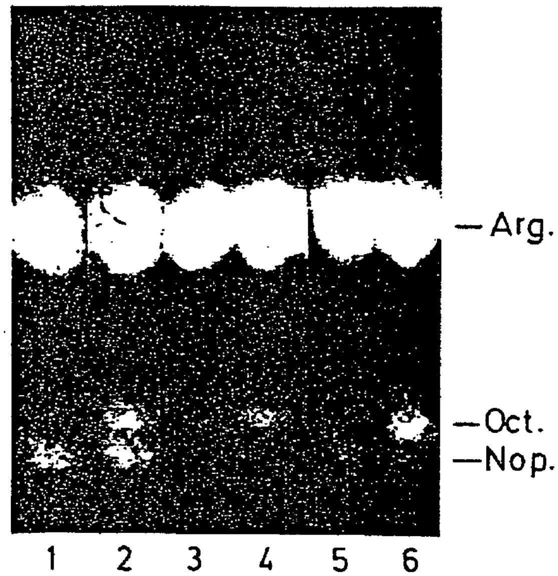


Fig. 3. Detection of octopine in tumors induced with *Agrobacterium* strains containing the mutant plasmids. 2 μl of extracts of tumor tissue before (lanes 1, 3, 5) and after (lanes 2, 4, 6) 1 h incubation were spotted onto Whatman 3MM paper and subjected to electrophoresis. Lanes 1 and 2: extracts obtained from tissue infected with *Agrobacterium* containing pGV2295; lanes 3 and 4: extracts obtained from tissue infected with *Agrobacterium* containing pGV2294; lanes 5 and 6: extracts obtained from tissue infected with *Agrobacterium* containing pGV2254.

Properties of pGV2253 and pGV2254. Agrobacterium strain containing either pGV2253 or pGV2254 were used to incite tumors on tobacco plants. These tumors synthesize nopaline and octopine (Figure 3), but no mannopine or agropine could be detected. This observation indicates that the deletion substitution of the small *Hind*III fragment 36a abolishes the synthesis of mannopine and agropine.

Morover, since the sequences between the end of the nopaline T-DNA (position −305) and the *Bcl*I site (position −261) have been deleted and replaced by the Sm^R gene of pR702, the 5′-flanking region of the nopaline synthase gene in this construction is separated from TR sequences located to the right (in pGV2253) or to the left (in pGV2254), by the Sm^R insert fragment. Therefore, all the sequences involved in the *in vivo* expression of the nopaline gene must lie within the 5′-flanking region between the start of transcription and the *Bcl*I site (position −261).

Discussion

Most of the understanding of the regulatory events controlling gene expression in higher eukaryotes is derived from studies with animal viruses. Several eukaryotic promoters have been examined both by DNA sequencing and by *in vitro* and *in vivo* analysis of mutants. These studies have led to the identification of the so-called Goldberg-Hogness or TATA box, a signal that is involved in the precise positioning of 5′ RNA ends of genes transcribed by RNA polymerase II (Breathnach and Chambon, 1981; Shenk, 1981). Although the TATA box seems to be both necessary and sufficient for

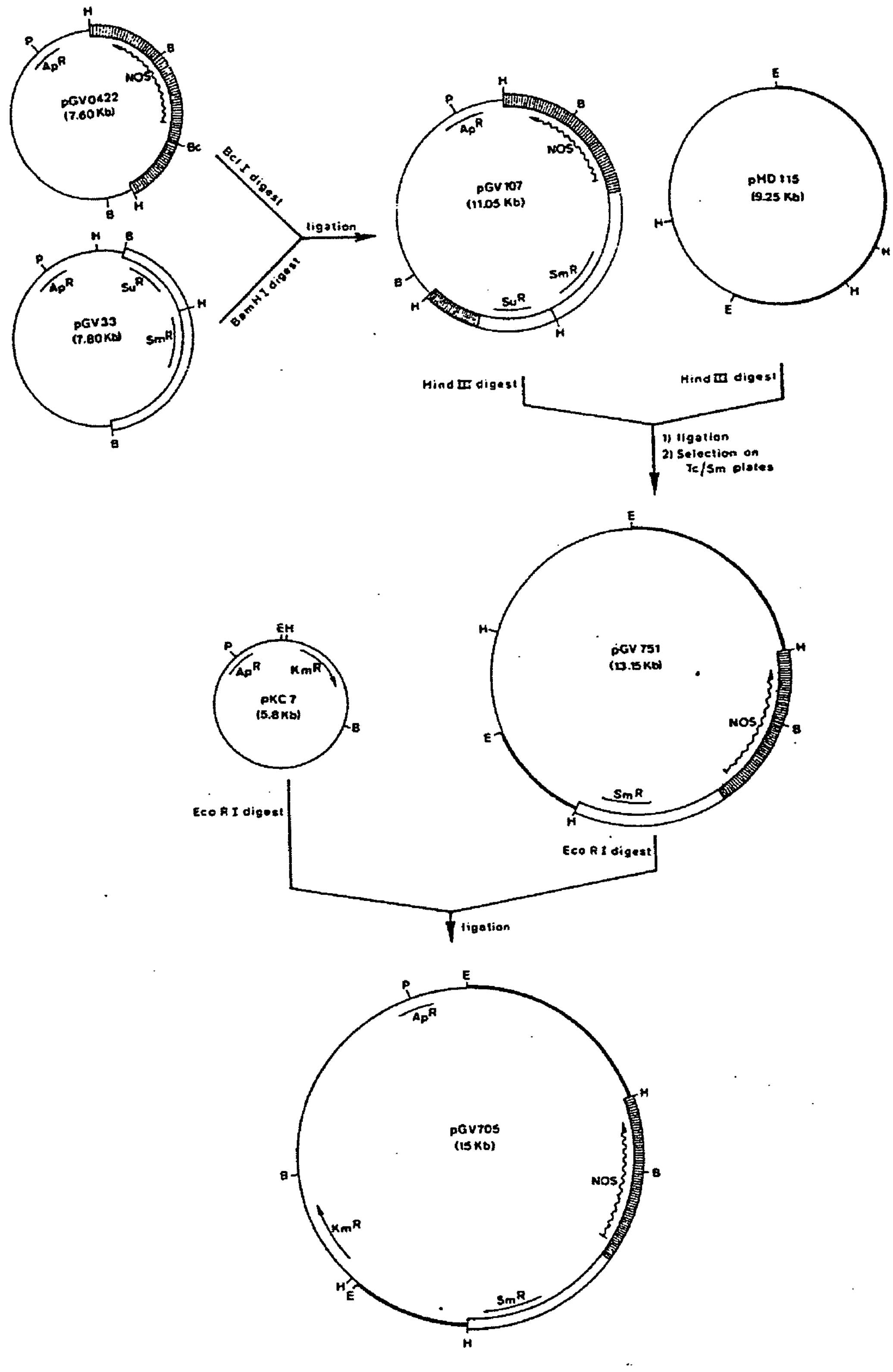


Fig. 4. Construction of the intermediate vector pGV705. Plasmids pGV0422 was linearized with *Bcl*I and ligated to *Bam*HI-digested pGV33. After transformation, recombinants were selected on Ap/Sm plates. One of the recombinants, pGV107, was digested with *Hind*III and ligated to *Hind*III-digested pHD115, containing the *Eco*RI fragment 12 of pTiAch5. After selection on Tc/Sm plates a recombinant, pGV751, was digested with *Eco*RI and ligated to *Eco*RI-digested pKC7, making it possible to use the mobilizing method described by Van Haute *et al.* (1983). Indeed, pGV751, a pACYC184 derivative, cannot be mobilized by pGJ28 and R64*drd*11.

Table I. Bacterial strains and plasmids

	Antibiotic resistance	Characteristics	Dimension (kb)	Origin
Strains				
E. coli				
K514		*thr leu thi hsd*R		Colson *et al.* (1965)
A. tumefaciens				
GV3101		Rif^R derivative of C58, cured for pTiC58		Van Larebeke *et al.* (1974)
GV3105		Ery^R Cml^R derivative of C58, cured for pTiC58		Holsters *et al.* (1980)
Plasmids				
pKC7	Ap Km	*Hind*III-*Bam*HI of Tn*5* in pBR322	5.8	Rao and Rodgers (1979)
pGV0153	Ap	*Bam*HI-8 of pTiAch5 in pBR322	11.6	De Vos *et al.* (1981)
pGV0201	Ap	*Hind*III-1 of pTiAch5 in pBR322	16.9	De Vos *et al.* (1981)
pGV0422	Ap	*Hind*III-23 of pTiC58 in pBR322	7.6	Depicker *et al.* (1980)
pGV705	Ap Km Sm	*Hind*III fragment containing the *nos* gene and Sm/Sp marker of R702 in *Eco*RI-12	15	This work
pGV706	Ap Km Sm	*Hind*III fragment containing the *nos* gene and Sm/Sp marker of R702 in *Eco*RI-12, but in opposite direction	15	This work
pGV717	Ap	*Hind*III-*Bam*HI fragment of gcl rGV1-1 in pBR322	5.1	Holsters *et al.* (1983)
pAGV828	Ap	*Bam*HI-*Sma*I of pGV99 in pBR322	5.25	Herrera-Estrella *et al.* (1983)
pGV761	Ap Km	*Cla*I-*Acc*I of pKC7 in pAGV828	6.7	This work
pGV762	Ap Km	*Taq*I-*Bam*HI of pGV717 in pGV761	6.35	This work
pGV763	Ap Km	*Hpa*II-*Bam*HI of pGV717 in pGV761	6.5	This work
pGV33	Ap Sm/Sp Su	3.5 kb *Bam*HI fragment of R702 in pBR322	7.7	J. Leemans
pHD115	Tc	*Eco*RI-12 fragment of pTiAch5 in pACY184	9.25	J. Velten
R702	Km Sm/Sp Tc Su Hg	P-type plasmid	69.0	Hedges and Jacobs (1974)
R64drd11	Tc Sm	Iα-type plasmid, transfer-derepressed derivative of R64	109.0	Meynell and Datta (1967)
pGJ28	Km/Nm	Cda^+ Ida^+ ColD replicon carrying ColE1 *mob* and *bom*	9.7	Van Haute *et al.* (1983)
pGV3100	–	pTiC58, derepressed for autotransfer	212	Holsters *et al.* (1980)
pGV3300	Ap	pGV3100::Tn*1*	215	Joos *et al.* (1983)
pGV3305	Ap	pGV3100::Tn*1*	215	D. Inzé
pTiB6S3Tra^c		pTiB6S3, derepressed for autotransfer	192	Petit *et al.* (1978)

accurate initiation of transcription *in vitro* (Corden *et al.*, 1980; Wasylyk *et al.*, 1980), regions further upstream are required for efficient *in vivo* transcription (Grosschedl and Birnstiel, 1980; Benoist and Chambon, 1980; McKnight *et al.*, 1981; Grosveld *et al.*, 1982; Weiher *et al.*, 1983). Recently, a detailed analysis of the promoter of the herpes simplex thymidine kinase (TK) gene (McKnight and Kingsbury, 1982) resulted in an identification of three essential regions within 105 bp upstream of the RNA initiation site.

In higher plants, on the contrary, little is known about sequence signals controlling gene expression. In octopine and nopaline crown gall tumor tissues, the T-DNA is transcribed by RNA polymerase II (Willmitzer *et al.*, 1981a), and encodes a set of well-defined polyadenylated transcripts. Therefore, the T-DNA genes can serve as models for defining transcriptional and translational control sequences in nuclear, protein-coding plant genes. In a first approach, we have attempted to determine which are the minimal 5′ upstream sequences involved in the *in vivo* expression of these opine genes. Deletion of sequences upstream of position −170 of the octopine synthase gene greatly reduces or abolishes the gene expression, while deletion of sequences upstream of position −294 does not interfere with a wild-type level of gene expression. In this sequence of 125 bp an essential region controlling the expression of the octopine synthase gene might be located. Also in the case of the nopaline synthase gene, the 5′ sequences downstream of position −261 contain all the information necessary for the *in vivo* expression of this gene. Therefore, the estimated maximum size of the octopine and nopaline synthase gene promoters are 295 bp and 261 bp, respectively. Although the DNA sequences directly involved in the expression of the opine synthase genes in plant cells are not defined in this study, and identification of these sequences could help in the elucidation of the mechanisms of plant cellular gene control, the results described above clearly demonstrate that the expression of octopine and nopaline synthase genes is

determined directly by their 5′ upstream flanking sequences and is independent of the direct vicinity of the plant DNA sequences.

Materials and methods

Bacterial strains and plasmids

Bacterial strains and plasmids are listed in Table I.

Media and culture conditions

Luria broth (LB) and minimal A (minA) media were as described (Miller, 1972). Nitrogen-free medium for the use of octopine or nopaline as sole nitrogen source were as described (Bomhoff *et al.*, 1976). *E. coli* cultures were grown at 37°C and *A. tumefaciens* at 28°C. Antibiotic concentrations used for *E. coli* and *A. tumefaciens* were respectively, carbenicillin (Cb), 100 μg/ml; streptomycin (Sm), 20 μg/ml and 300 μg/ml; spectinomycin (Sp), 50 μg/ml and 100 μg/ml; kanamycin (Km), 25 μg/ml; rifampicin (Rif), 100 μg/ml; erythromycin (Ery), 50 μg/ml for *Agrobacterium*; chloramphenicol (Cml), 25 μg/ml for *Agrobacterium*.

Plasmid isolation

Plasmids were prepared from *E. coli* by density gradient centrifugation in a CsCl-ethidium bromide gradient of cleared SDS lysates (Betlach *et al.*, 1976). For screening of recombinant plasmids, plasmid DNA was obtained from 10 ml cultures as described (Klein *et al.*, 1980).

DNA analysis

Restriction enzyme analysis, agarose gel electrophoresis, conditions for DNA ligation and transformation of competent *E. coli* were as described (Depicker *et al.*, 1980). DNA fragments were extracted from low-gelling agarose gels as described (Wieslander, 1979). Total DNA of Ti plasmid-containing *Agrobacterium* strains was prepared, digested, separated on agarose gel, transferred to nitrocellulose paper, and hybridized against radioactively labeled recombinant plasmids as described (Dhaese *et al.*, 1979).

Induction and culture of crown gall tumors

Sterile 1-month-old tobacco plants (Wisconsin 38 or SR1) were decapitated and infected with freshly grown agrobacteria. Three weeks later, tumors were excised and transferred to hormone-free Murashige and Skoog medium (Murashige and Skoog, 1962) containing sucrose (30 g/l) and 0.5 mg/ml HR756 (Hoechst A.G.). The tumor tissues, transferred every month, were usually free of bacteria after three transfers, and were further cultivated on antibiotic-free Murashige and Skoog medium. Sunflower hypocotyl segments were inoculated as described by Petit and Tempé (1978).

Detection of opines in plant tumor tissue

Octopine and nopaline detection. The presence of octopine or nopaline in tumor tissue was tested as described by Leemans *et al.* (1981). Octopine or nopaline synthase activity were determined *in vitro* according to Otten and Schilperoort (1978).

Agropine and mannopine detection. Agropine and mannopine were detected in tumor tissue as described by Leemans *et al.* (1981).

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XI. RELATED PROCEEDINGS APPENDIX

NONE.

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